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VIRUS-CELL INTERACTIONS

High Prevalence of Infectious Adeno-associated Virus (AAV) in Human Peripheral Blood Mononuclear Cells Indicative of T Lymphocytes as Sites of AAV Persistence

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ABSTRACT Seroepidemiology shows that infections with adeno-associated virus (AAV) are widespread, but diverse AAV serotypes isolated from humans or nonhuman primates have so far not been proven to be causes of human disease. In view of the increasing success of AAV-derived vectors in human gene therapy, definition of the *in vivo* sites of wild-type AAV persistence and the clinical consequences of its reactivation is becoming increasingly urgent. Here, we identify the presumed cell type for AAV persistence in the human host by highly sensitive AAV PCRs developed for the full spectrum of human AAV serotypes. In genomic-DNA samples from leukocytes of 243 healthy blood donors, 34% were found to be AAV positive, predominantly AAV type 2 (AAV2) (77%), AAV5 (19%), and additional serotypes. Roughly 11% of the blood donors had mixed AAV infections. AAV prevalence was dramatically increased in immunosuppressed patients, 76% of whom were AAV positive. Of these, at least 45% displayed mixed infections. Follow-up of single blood donors over 2 years allowed repeated detection of the initial and/or additional AAV serotypes, suggestive of fluctuating, persistent infection. Leukocyte separation revealed that AAV resided in $CD3^+$ T lymphocytes, perceived as the putative *in vivo* site of AAV persistence. Moreover, infectious AAVs of various serotypes could be rescued and propagated from numerous samples. The high prevalence and broad spectrum of human AAVs in leukocytes closely follow AAV seroepidemiology. Immunosuppression obviously enhances AAV replication in parallel with activation of human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6), reminiscent of herpesvirus-induced AAV activation.

IMPORTANCE Adeno-associated virus is viewed as apathogenic and replication defective, requiring coinfection with adenovirus or herpesvirus for productive infection. *In vivo* persistence of a defective virus requires latency in specialized cell types to escape the host immune response until viral spread becomes possible. Reactivation from latency can be induced by diverse stimuli, including infections, typically induced upon host immunosuppression. We show for the first time that infectious AAV is highly prevalent in human leukocytes, specifically T lymphocytes, and that AAV is strongly amplified upon immunosuppression, along with reactivation of latent human herpesviruses. In the absence of an animal model to study the AAV life cycle, our findings in the human host will advance the understanding of AAV latency, reactivation, and *in vivo* pathogenesis.

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Adeno-associated viruses (AAVs) are small, replication-defective DNA viruses originally isolated as contaminants of human or simian adenovirus (Ad) stocks (1–4). Only AAV type 5 (AAV5) and AAV9 were isolated directly from human tissue (5, 6), whereas most other AAV serotypes stem from nonhuman primates, some after prior adenovirus infection (7, 8). Depending on age, sex, and geographical region, up to 80% of the human population display antibodies against the human AAV serotypes 1 to 3 and AAV5 (9–14). Direct comparison of serotype-specific IgGs showed a seroprevalence for AAV1 and AAV2 of around 70%, whereas seroprevalences for AAV5 (40%), AAV6 (46%), AAV8 (38%), and AAV9 (47%) were generally lower (15, 16). The development of neutralizing antibodies against AAV1, -2, -5, and -8 followed the same pattern (15, 16). Seroepidemiology indicates that AAV infections are ubiquitous in human subjects, but the clinical consequences are largely unknown. Indeed, AAV is generally considered apathogenic, since it has not yet been correlated unequivocally with human disease.

In cell culture, AAV2 displays a unique biphasic life cycle that has been studied in detail over the years. AAV latency is characterized by episomal persistence or chromosomal integration (17–20) until an unrelated helper virus initiates productive AAV replication. Besides adenovirus (1), all herpesvirus genera tested until now, including herpes simplex virus (HSV) (21), varicella-zoster virus (VZV) (13), human cytomegalovirus (HCMV) (13, 22), Epstein-Barr virus (EBV) (13), and human herpesvirus 6 (HHV-6) (23), were shown to support AAV replication. *In vivo*, AAV2 DNA was detected in 9 out of 175 (5%) human tissue samples, predominantly as episomes, but a single integration event was described on chromosome 1 (20).

Although cell receptors were identified for many AAV serotypes, and susceptible cell types were described, little is known about the primary routes of AAV infection. AAV seroconversion during early childhood is suggestive of respiratory transmission, along with adenovirus infection (14, 16), though evidence for a direct link is lacking. In addition, sexual transmission has been postulated, as AAV2 DNA was isolated from the cervix, semen, and spontaneous abortions (24–26). In diverse human tissue samples with and without pathologies, AAV DNA was detected at various frequencies (6, 20, 27). Common cell types present in all mixed-cell tissue samples represent peripheral blood cells. In line with this reasoning is the early PCR identification of AAV2 DNA in 2 out of 55 samples of peripheral blood leukocytes (28). Furthermore, AAV DNA was detected in peripheral blood mononuclear cells (PBMCs) of rhesus monkeys 21 days after iatrogenic AAV infection, alone or together with adenovirus (29). However, a striking discrepancy remains between the reported high seroprevalence and the occasional detection of AAV in specific cell types or tissues.

These findings led to our hypothesis that human AAVs regularly reside in PBMCs. This assumption was particularly intriguing, since PBMCs also represent sites of latency and/or active replication of AAV helper viruses: Ad, CMV, EBV, and HHV-6 (30–34). All of them are prone to reactivation in immunosuppressed patients, often with adverse outcomes (35–38). Here, we show for the first time that a broad spectrum of replication-competent AAV serotypes persist in human leukocytes, specifically in T lymphocytes, at relative frequencies consistent with reported AAV seroepidemiology. Furthermore, immunosuppression boosts the rates of AAV detection and of mixed infections, indicative of AAV reactivation.

RESULTS

Establishment of a highly sensitive AAV-specific nested PCR. Wild-type AAV resides and replicates in the cell nucleus. Therefore, PCR detection of AAV DNA genomes had to be performed against a background of total genomic DNA extracted from the cell of choice. A conserved target sequence identified between nucleotides (nt) 2350 and 2899 of AAV2 *cap* was chosen for PCR detection of most AAV serotypes

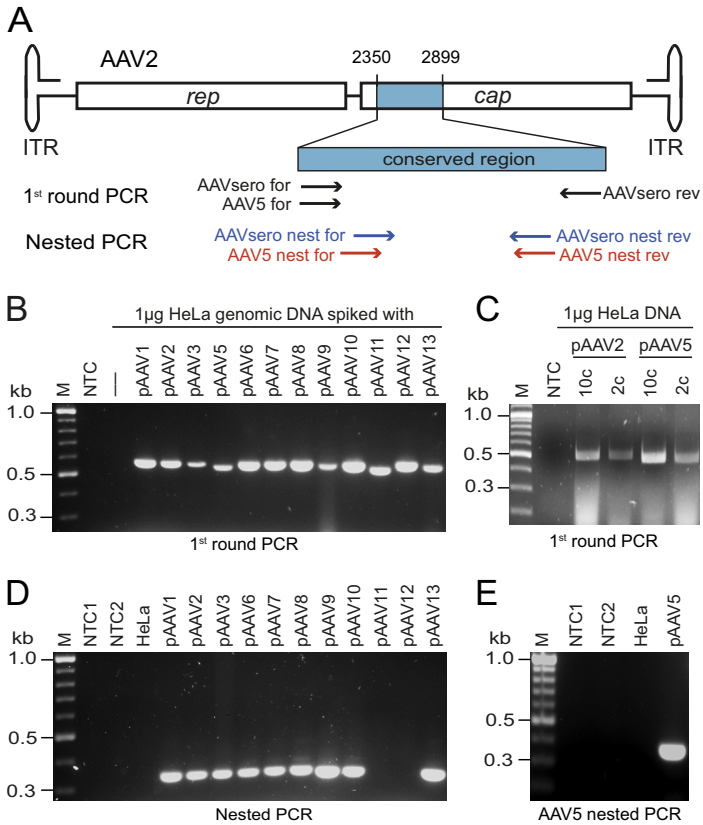

FIG 1 Highly sensitive nested PCR for the detection of AAV serotypes 1 to 13. (A) Schematic representation of the AAV genome, with *rep* and *cap* genes flanked by inverted terminal repeats (ITR). The highly conserved region within the *cap* gene of AAV2 (nt 2350 to 2899) is enlarged. All the primers are depicted by arrows. AAVsero for and AAVsero rev bind to all AAV serotypes (1 to 13) except AAV5. Primer AAV5 for is used to amplify AAV5 in combination with AAVsero rev. AAVsero nest for and AAVsero nest rev bind to AAV1 to -3, -6 to -10, and -13. AAV5 is detected by the primer pair AAV5 nest for and AAV5 nest rev. (B) Validation of 1st-round PCR on 1 μg genomic DNA from HeLa cells alone or spiked with 10 copies of linearized plasmids harboring *cap* genes of the depicted AAV serotypes. M, molecular size marker; NTC, nontemplate control. The 1st-round PCR product ranged between 532 bp and 553 bp depending on the serotype. (C) First-round PCR performed as for panel B but on 2 copies of AAV2 or AAV5 *cap*-carrying plasmids. (D) Nested PCR with a 1:100 dilution of 1 μl 1st-round PCR mixture as depicted in panel B as a template. NTC1, DNase-free water; NTC2, amplification of the nontemplate control of the 1st-round PCR. Product lengths ranged from 329 to 332 bp. (E) AAV5 nested PCR with AAV5 nested primers amplifying a product of 364 bp.

(Fig. 1A). The selected primers display between 90 and 100% homology to AAV serotypes 1 to 13. For AAV5, a specific forward primer was designed, as described in Materials and Methods. To validate the PCR conditions, 1 μg of genomic HeLa DNA, roughly equivalent to 1.7×10^5 diploid cells, was spiked with 10 plasmid copies of each AAV serotype, leading to defined PCR bands between 532 bp and 553 bp (Fig. 1B). AAV2 and -5 were also tested at 2 copies per 1 μg genomic DNA (Fig. 1C). AAV4 was not analyzed, since it is not of human origin. Antibodies reactive to AAV4 have never been found in human subjects (3, 9), and AAV4 DNA has never been identified in human tissues (6, 20, 39). To increase the sensitivity of AAV detection, a nested PCR was established, spanning nucleotides 2520 to 2848 of AAV2, with primer pairs that match AAV1 to -3, -6, -8, and -13 100% and show a single mismatch in one of the primers for AAV7, -9, and -10. The nested reverse primer showed mismatches of more than 10 bases for AAV4, -11, and -12 (data not shown). Since these AAVs are not of human origin, their absence was expected. For AAV5, a specific nested primer pair was designed. Nested AAV PCRs led to product lengths between 329 bp and 364 bp, depending on the serotype (Fig. 1D and E). The nested AAV PCRs are highly sensitive, allowing the detection of 1 AAV genome per 10^5 cells.

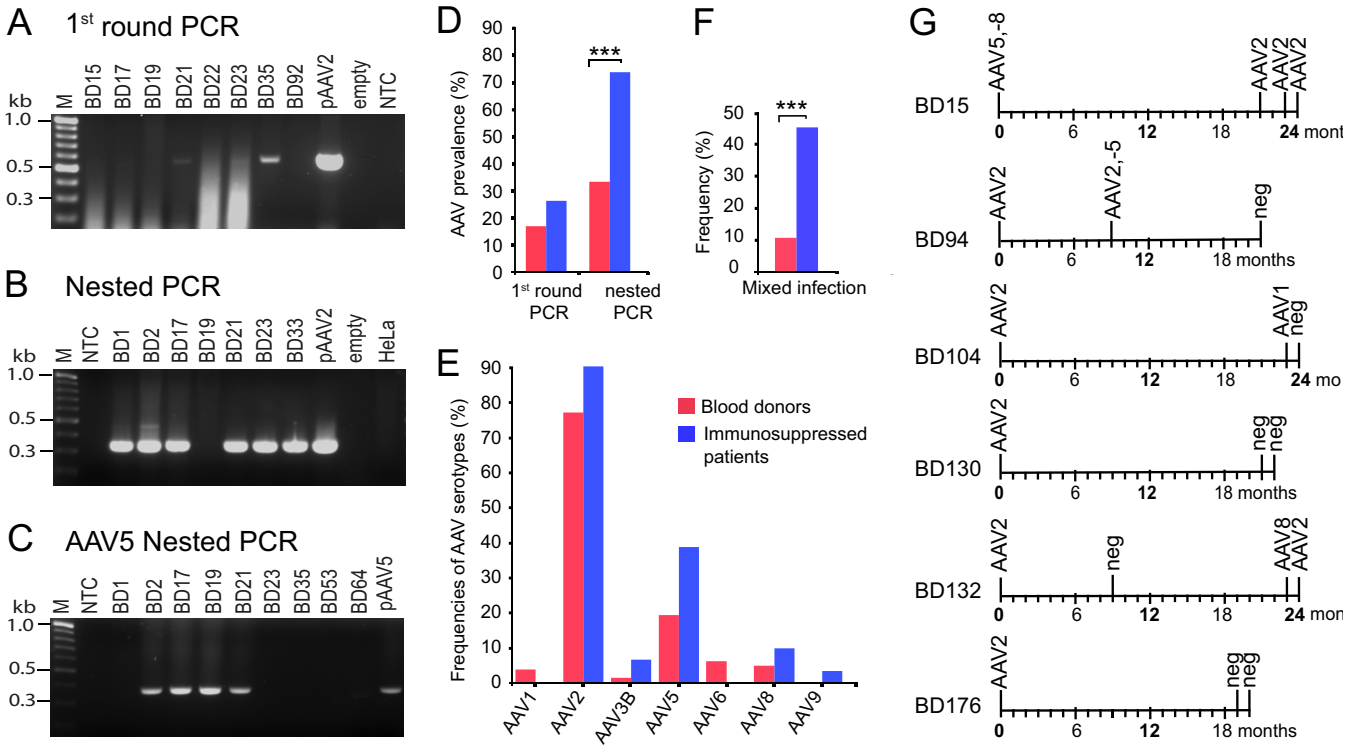

FIG 2 Detection of AAV in PBMCs of blood donors. (A) Results of 1st-round PCR on 1 μg genomic DNA extracted from PBMCs of blood donors (BD) as indicated by the numbers. A representative gel shows amplicons of the expected lengths between 532 and 553 bp. M, molecular size marker; pAAV2, positive control comprising 10 copies of an AAV2 *cap*-expressing plasmid on the background of 1 μg genomic DNA; NTC, nontemplate control. Note that sample selection for panels A, B, and C varied for technical reasons. (B) Representative gel from nested PCR with AAVsero nest for and AAVsero nest rev primers on blood samples from donors. The nested-PCR products ranged between 329 and 332 bp. (C) Representative gel of AAV5 nested-PCR products (364 bp). (D) Prevalence of AAV already detected by 1st-round and nested PCR in healthy blood donors and immunosuppressed patients. (Figure 3 shows a representative gel from PCRs displaying samples of immunosuppressed patients.) ***, Highly significant ($P < 0.00001$). (E) Relative distribution of AAV serotypes in blood donors and immunosuppressed patients. AAV serotypes were identified by DNA sequence analysis of purified AAV PCR products. Note that all AAV1 DNA sequences share a silent mutation at position 2774, where T is substituted for C of the published AAV1 sequence (NC_002077.1). (F) Frequencies of mixed infections with more than one AAV serotype. ***, high significance ($P < 0.0001$). (G) Follow-up (24 months) of AAV persistence. Time lines and PCR results for six blood donors tested 3 or 4 times are depicted. The donors' anonymous codes are shown on the left. The serotype identified is depicted at the respective time point. "neg" indicates that a sample was tested but found negative for AAV at the respective time point.

AAV serotypes in leukocytes of blood donors. Blood samples from 243 healthy blood donors were collected, and genomic DNA was extracted from buffy coats or PBMCs to analyze the prevalence of AAV. PCR results were considered only when at least two negative controls (H_2O and HeLa cell DNA) and two positive controls (HeLa cell DNA spiked with 10 plasmid copies of AAV2 or AAV5) reacted as expected. To ensure that genomic DNA extracted from buffy coats did not contain any PCR inhibitors, 250 ng to 1 μg genomic DNAs of six blindly chosen buffy coats were spiked with 10, 10^2, or 10^5 copies of a control plasmid carrying AAV2 *cap*. Parallel PCRs with spiked genomic DNAs of HeLa cells or with control plasmids alone were performed. There was no obvious difference in signal intensities between samples analyzed on the background of buffy coat DNA, HeLa cell DNA, or H_2O, confirming that the extracted genomic DNA was sufficiently pure (data not shown). From 225 samples from healthy blood donors, 1 μg of genomic DNA was analyzed. An additional 18 samples with lower DNA yields (0.3 to 0.9 μg) were tested similarly. In total, 42 samples were already AAV positive in 1st-round PCR (17%). Nested PCRs were carried out with the primer pair AAVsero nested or AAV5 nested. A total of 83 out of 243 blood donor samples proved to contain AAV DNA (34%) (Fig. 2A to D). A series of AAV-positive samples were tested more than once, including all the controls, proving that the initial PCR results were reproducible (data not shown). PCR fragments of all AAV-positive samples were purified for DNA sequence analysis to identify the AAV serotype concerned. As shown in Fig. 2E,

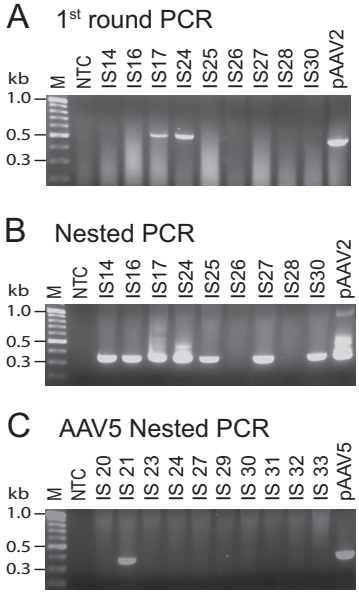

FIG 3 Detection of AAV in PBMCs of immunosuppressed patients. PBMCs isolated from 41 immunosuppressed patients were tested for the presence of AAV DNA, using an AAV PCR assay as shown in Fig. 2. (A) Representative gel of 1st-round PCR. (B) Representative gel of AAV nested PCR. (C) Representative gel of AAV5 nested PCR.

64 samples (77%) contained AAV2 and 16 (19%) contained AAV5. Occasional AAV isolates with 95 to 98% DNA sequence homology to published serotypes (AAV2, -3B, -6, -8, and -10) were detected. Mixed infections with more than one AAV serotype accounted for 11% (Fig. 2F).

AAV in leukocytes of immunosuppressed patients. AAV is replication defective, and some of the described helper viruses, CMV (22), EBV (13), and HHV-6 (23), establish latency in cells of the hematopoietic system and are frequently reactivated under immunosuppression. The high prevalence of AAV DNA in PBMCs of healthy blood donors prompted us to investigate AAV prevalence in PBMCs of immunosuppressed patients. Genomic DNA was extracted from buffy coats of 41 posttransplant patients under immunosuppressive therapy. The samples were PCR analyzed for AAV DNA as described above. Already in the 1st PCR round, 11 out of 41 samples (27%) scored positive (Fig. 3A). With nested AAVsero PCR, 29 samples scored positive, and with nested AAV5 PCR, 12 were positive. In cases of mixed infections (see below), 31 patients tested AAV positive (Fig. 3B and C). Taken together, AAV prevalence doubled in immunosuppressed patients (76%) compared to healthy blood donors (34%; $P < 0.000001$) (Fig. 2D). Also, the percentage of mixed infections was significantly higher in immunosuppressed patients (45%; $P < 0.0001$) than in blood donors (11%) (Fig. 2F). All AAV-positive samples were sequenced and often showed multiple superimposed DNA sequences of various serotypes. We therefore cloned and sequenced many of the PCR products and confirmed that, apart from a dominant serotype, mostly AAV2, various additional serotypes were present (Table 1). Although we cannot rule out the possibility that certain AAVs are underestimated in the case of mixed infections, the distribution of serotypes was comparable to that in healthy blood donors. Of all positive samples, 90% were found to be positive for AAV2, followed by AAV5 (39%) and occasionally other serotypes (Fig. 2E).

Persistence of AAV infection. The high prevalence of AAV infection in healthy blood donors accompanied by frequent mixed infections was suggestive of persistent infection. To provide further evidence for this assumption, some AAV-positive blood donors were tested repeatedly. Over a follow-up period of 2 years, 19 blood donors were tested repeatedly. Of these, 42% were confirmed as AAV positive ($n =$

TABLE 1 Distribution of AAV serotypes detected as single isolates and mixed with other serotypes in blood donors (BD) and immunosuppressed patients (IS)

	Single isolate		Mixed infection with another serotype(s)[b]		Total no. of isolates	
Serotype	BD[a]	IS	BD	IS	BD	IS
AAV1	2		1 (AAV5)		3	
AAV2	58	14	4 (AAV5)	1 (AAV3B)	64	28
			1 (AAV6)	1 (AAV3B, -5)		
			1 (AAV5, -8)	9 (AAV5)		
				2 (AAV8)		
				1 (AAV9)		
AAV3	1			1 (AAV2)	1	2
				1 (AAV2, -5)		
AAV5	8	2	1 (AAV1)	9 (AAV2)	16	12
			4 (AAV2)	1 (AAV2, -3B)		
			2 (AAV8)			
			1 (AAV2, -8)			
AAV6	4		1 (AAV2)		5	
AAV8	1	1	2 (AAV5)	2 (AAV2)	4	3
			1 (AAV2, -5)			
AAV9		1				1

[a]BD, blood donors; IS, immunosuppressed patients.
[b]The numbers indicate the numbers of isolates for the serotypes in parentheses.

19) (Table 2), and 33% of those tested for a third time were positive again ($n = 6$) (Fig. 2G). Two blood donors could be tested 4 times, and both were repeatedly positive. However, an individual's detected AAV serotype occasionally varied from sample to sample. This finding may reflect fluctuation of the virus load, particularly in cases of infections with more than one AAV serotype. As described above, mixed infections were found in 11% of healthy blood donors. The frequency of mixed infections was significantly higher in immunosuppressed patients, as mentioned previously (Fig. 2F).

Analysis of PBMCs for the presence of AAV helper viruses. The frequency of AAV detection in PBMCs combined with the significantly increased AAV prevalence under immunosuppression prompted us to search for an association with described AAV helper viruses known to persist latently in PBMC subpopulations. They include HCMV and HHV-6, known to be reactivated and to cause disease under immunosuppression.

TABLE 2 Follow-up PCR of some positive blood donors[a]

Sample no.	1st test result	Serotype(s)	Time interval (mo)	2nd test result	Serotype(s)
BD2	Positive	AAV2, -5	10	Positive	AAV2, -5
BD4	Positive	AAV5	24	Negative	
BD15	Positive	AAV5, -8	22	Positive	AAV2
BD19	Positive	AAV5	10	Negative	
BD21	Positive	AAV2, -5	22	Negative	
BD22	Positive	AAV6	24	Negative	
BD33	Positive	AAV2	9	Positive	AAV2
BD35	Positive	AAV8[b]	9	Negative	
BD48	Positive	AAV2	9	Positive	AAV1
BD89	Positive	AAV2	23	Negative	
BD94	Positive	AAV2	9	Positive	AAV2, -5
BD104	Positive	AAV2	23	Positive	AAV1
BD106	Positive	AAV2	9	Negative	
BD130	Positive	AAV2	21	Negative	
BD132	Positive	AAV2	9	Negative	
BD138	Positive	AAV5	21	Negative	
BD176	Positive	AAV2	19	Negative	
BD244	Positive	AAV2	1	Positive	AAV2
BD258	Positive	AAV2	2	Positive	AAV2, -5

[a]Nineteen positive blood donors were tested twice. The serotype detected each time is presented.
[b]95% homology to published sequence.

TABLE 3 Candidate helper virus prevalence in AAV-positive subjects

Helper virus	Prevalence (%)	
	Blood donors	Immunosuppressed patients
HCMV	<2 ($n = 46$)	15 ($n = 27$)
HHV-6	7 ($n = 71$)	14 ($n = 28$)

Percentages of HCMV and HHV-6 PCR-positive leukocytes from AAV-positive blood donors and immunosuppressed patients, respectively; *n*, number of samples.

In AAV-positive blood donors, HCMV DNA could not be detected in any of the examined leukocyte samples, as expected from healthy subjects, and a low PCR detection rate of HHV-6 (7%) reflects the results reported previously for larger patient cohorts analyzed under the same PCR conditions (40). In AAV-positive immunosuppressed patients compared to blood donors, the detection rate of HCMV DNA was significantly increased (14%; $P < 0.02$). Also, the HHV-6 detection rate was increased (14%), and three of four HCMV-positive samples were also positive for HHV-6 (Table 3).

Persistence of AAV in CD3$^+$ T lymphocytes. PBMCs represent a mixed cell population. To determine the cellular subtype of AAV persistence, PBMCs were purified from 50-ml freshly drawn blood samples from five previously tested, AAV-positive, healthy blood donors. The PBMCs were separated by magnetically activated cell sorting (MACS) using CD14 beads to separate monocytes (optional) or CD3 beads to select for T lymphocytes. The bead-selected fractions were analyzed by nested AAV PCR, followed by DNA sequence analysis (Fig. 4). In four samples, AAV2 DNA was detected exclusively in the CD3$^+$ fraction. In one of these (BD258), AAV5 DNA was also detected, surprisingly, in both CD3$^+$ and CD3$^-$ fractions. The DNA sequence of the 1st-round and nested-PCR products showed that the CD3$^-$ fraction contained AAV5 exclusively, while the CD3$^+$ fraction contained both AAV2 and AAV5. Sample BD104 was negative for AAV, although it had tested positive 24 and 2 months before (Fig. 2G). None of the CD3$^+$ samples was positive for HCMV or HHV-6. AAV copy numbers were determined in CD3$^+$ samples by quantitative PCR (qPCR) for AAV2, resulting in one AAV single-stranded DNA (ssDNA) copy in 5×10^2 to 2×10^4 CD3$^+$ cells (data not shown).

Rescue of infectious AAV. To prove that the AAV genomes detected in lymphocytes stem from replication-competent virus, we rescued infectious virus and quantified AAV replication in secondary-infection assays. Total genomic-DNA samples from individual blood donors or patients were transfected into 293 cells together with a plasmid supplying adenoviral helper functions. To test for infectious AAV particles, cell-free supernatants thereof were used for secondary infection of adenovirus-infected HeLa cells. We were able to rescue and replicate AAV from genomic DNA of 13 out of 21 previously tested AAV-positive blood donors, of all 4 AAV-positive MACS-purified T-lymphocyte samples, and of 4 out of 9 samples from immunosuppressed patients. Genomic DNA from three blood donors that tested AAV negative previously served as negative controls. AAV replication after second-round infection was verified by analysis of Rep and Cap expression on Western blots (Fig. 5A), and AAV titers were quantified by qPCR (Fig. 5B). In view of the very low abundance of AAV in the original samples, it could not be decided whether the occasional failure to rescue replicating AAV from leukocytes was due to the presence of defective virus or insufficient AAV replication. The rescue protocol had been optimized for AAV2 replication in Ad type 5 (Ad5)-transfected 293 cells or Ad2-infected HeLa cells and may not be optimal for other AAV serotypes. Notably, two of the rescued AAVs verified as AAV5 by AAV5-specific nested PCR showed titers 5 log units lower than those of AAV2 in the same sample. Our previous experience with propagation of wild-type AAV2 or AAV5 stocks confirmed a reduced replication capacity of AAV5 in 293 or HeLa cells (D. Hüser, unpublished data). Moreover, the two samples with the lowest replication rates (IS30 and IS36) (Fig. 5B) were identified as AAV8 or AAV9.

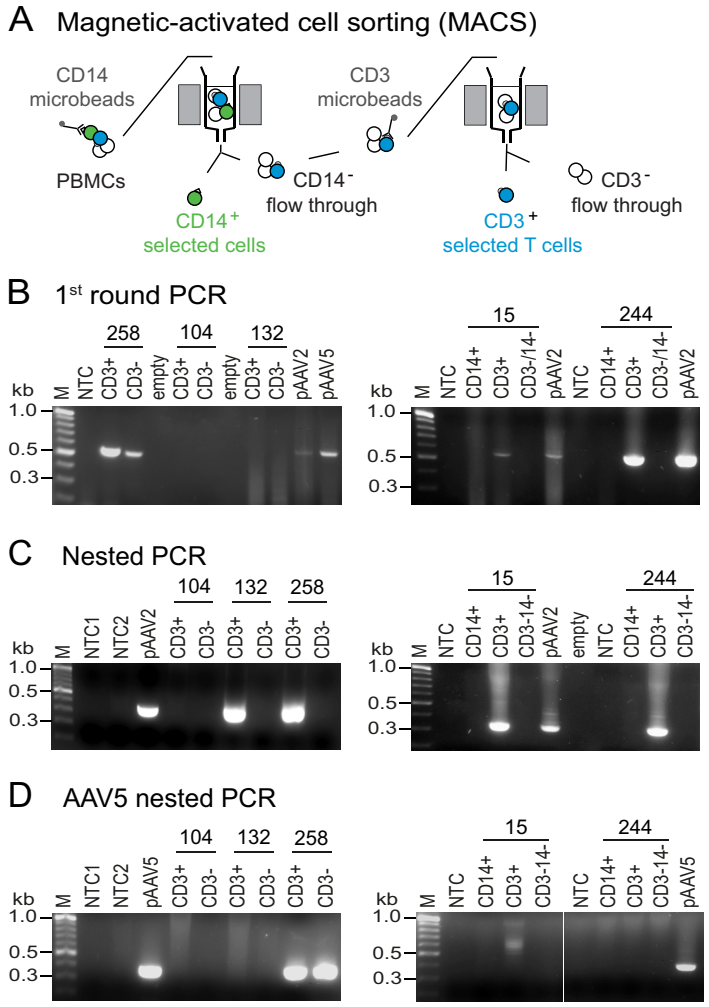

FIG 4 Identification of CD3$^+$ T lymphocytes as sites of AAV persistence. (A) Schematic representation of MACS as performed in this study. Freshly drawn blood samples were collected from 5 previously tested, AAV-positive blood donors. PBMCs were separated by Ficoll-Paque density gradient centrifugation. Two samples (BD15 and BD244) were separated into CD14$^+$, CD3$^+$, and CD3$^-$/CD14$^-$ fractions. Three samples (BD132, BD104, and BD258) were separated into a CD3$^+$ fraction and a CD3$^-$ fraction. Genomic DNA was isolated, and PCR was performed as described in Materials and Methods. (B and C) First-round PCR (B) and nested PCR (C) with AAVsero nested primers. (D) AAV5 nested PCR.

DISCUSSION

In spite of the ubiquity of AAV DNA detected in diverse human tissues (6, 20, 27), a unique target cell type for regular AAV persistence *in vivo* has not yet been identified. In the absence of a manageable animal model, the *in vivo* biology of wild-type AAV infection remains largely unknown. Our study identifies for the first time human leukocytes, particularly T lymphocytes, as regular sites for long-term persistence of replication-competent AAVs of various human serotypes. Moreover, we show for the first time that the prevalence of infectious AAV is significantly increased in immunosuppressed patients, suggestive of AAV reactivation in parallel with the reactivation of known helper viruses. Predominantly herpesviruses, but also adenoviruses, are activated upon immunosuppression, often with adverse clinical outcomes (35, 41–44). It will be challenging to study mutual interactions in their *in vivo* pathogeneses. Last but not least, the increasing application of AAV-based vectors for gene therapy calls for more insight into the natural course of wild-type AAV infection in order to evaluate the safety of AAV vectors in clinical use.

Fluctuating AAV detection during long-term persistence. The strongest evidence for human leukocytes being the preferential site of AAV persistence comes from

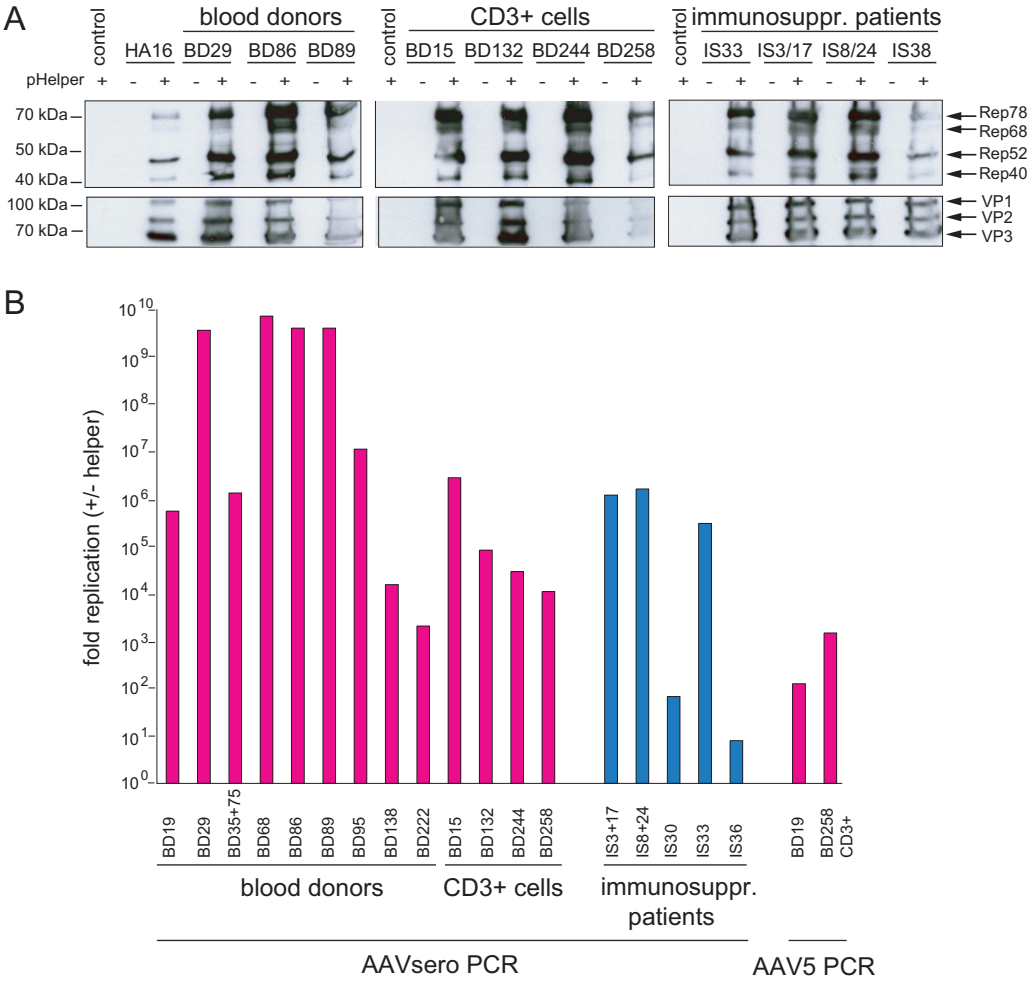

FIG 5 Rescue of infectious AAV from genomic DNA of blood donors or immunosuppressed patients. Human genomic DNA was transfected into 293 cells along with a plasmid providing adenoviral helper functions (pHelper), as indicated. For second-round amplification, supernatants containing newly generated AAV particles were used to infect Ad2-infected HeLa cells. (A) Second-round analysis of AAV Rep and Cap expression on Western blots detected with MAb 303.9 or B1, respectively. Previously tested AAV-negative blood donor DNAs served as negative controls. Control DNA was spiked with 6 ng (equaling 10^3 cells) DNA of HA16, a HeLa cell line carrying integrated, rescuable AAV2 genomes, serving as a positive control. (B) AAV genomic particle yields were analyzed in purified cell extracts by qPCR. Shown are ratios of titers obtained with versus without pHelper. AAV5 PCR was carried out as a nested PCR to ensure high specificity for AAV5.

the repeated detection of AAV in blood donors over follow-up periods of up to 2 years. Often, however, a donor was not AAV positive at all time points or presented with another AAV serotype later. These data are highly suggestive of fluctuating AAV levels. Our PCR assay detects 1 AAV copy in genomic DNA of 10^5 diploid cells. When AAV levels drop below this limit, AAV persistence escapes detection. Reinfection with the same AAV serotype appears highly unlikely in view of the described high prevalence of neutralizing antibodies against AAV serotypes (14, 15), unchanged even under immunosuppression (45). Repeated AAV detection during the follow-up of individual subjects is therefore considered evidence of fluctuating, persistent infection with different AAV serotypes. The significantly higher frequency of mixed infection in immunosuppressed patients than in healthy donors supports this notion and implies that coexisting persistent AAV serotypes can be reactivated.

Prevalence of AAV serotypes in human subjects. The relative proportions of different AAVs revealed that AAV2 is the most prevalent serotype. AAV2 is detected in 77% of AAV-positive healthy blood donors and 90% of patients under immunosuppression, consistent with the reported high seroprevalence of AAV2 in the human population (14, 15). In contrast, AAV1 DNA was detected rarely, in spite of a comparable

seroprevalence (15). AAV1 and -2 capsids are highly conserved, so cross-reactivity likely explains the effect. The nearly perfect correlation of anti-AAV2 and anti-AAV1 IgG positivity in over 200 blood samples further underscores this notion (15). Our findings are in line with those of a previous study showing that AAV2 is the most prevalent serotype in various human tissue samples, while AAVs of clade A, including AAV1 and AAV6, were found in only 3 out of 259 human samples (6). For the first time, AAV3B was isolated, a human-derived serotype not described before in human tissues (6, 20). The second most prevalent AAV in human blood samples was AAV5, detected in 19% of blood donors and 39% of immunosuppressed patients. Indeed, this was the first detection of infectious AAV5 after its initial identification (5). AAV6 DNA was detected rarely (6% of blood donors). Again, this is at variance with the reported seroprevalence of 46%. Since AAV6 is highly similar to AAV1, the discrepancy is likely due to cross-reactivity (15). Neither AAV4 nor AAV7 was found, confirming the described simian origin (6). AAV8 DNA was identified in 5% of blood donors and in 10% of immunosuppressed patients. AAV9, first isolated from 3 out of 259 human tissue samples (6), was isolated once from an immunosuppressed patient. In a recent report, 70% of cytokine-primed $CD34^+$ peripheral blood stem cell (PBSC) samples were found to represent or to share high homology with wild-type AAV9 (39). This high detection rate of a single, only sporadically detected human serotype is currently unexplained, unless AAV9 displays a highly selective tropism for $CD34^+$ cells. Follow-up studies with sets of AAV serotype-specific primers or conducted by whole-genome sequencing will help to differentiate the unexpectedly high frequencies of mixed AAV infections in PBMCs.

T lymphocytes as sites of AAV persistence. We show here that AAV2, the most prevalent serotype, resides in $CD3^+$ T lymphocytes rather than in monocytes or B lymphocytes. This novel finding is consistent with the previous detection of AAV in blood-perfused human or primate mixed-cell tissue samples, where the AAV-positive cell type was not defined (6). Although we cannot exclude the possibility of other sites of AAV persistence, the high prevalence of infectious AAVs in human PBMCs perfectly correlates with the described seroepidemiology. Furthermore, the increased AAV prevalence in PBMCs of immunosuppressed patients compared to healthy blood donors, described here for the first time, correlates with the reported higher frequency of AAV capsid-specific T cells in transplant patients compared to healthy controls (45). Human herpesviruses are regularly activated upon immunosuppression and, as established helper viruses, may lead to AAV replication. HCMV could have represented an obvious candidate but had not been shown to persist in T lymphocytes (30). Persistence in T lymphocytes is known for HHV-6 (46), which is highly prevalent in the human population, with seropositivity rates of up to 90% (47). Interestingly, HHV-6 carries a functional AAV *rep* gene homologue that HHV-6 likely acquired during coinfection with AAV (48). AAV *rep* homologues have also been acquired by herpesviruses of other species, such as rat cytomegalovirus (49) and a recently identified bat herpesvirus (50). These findings support the notion that the *in vivo* interaction between AAV and herpes group viruses across species may be more common than previously anticipated. Nonetheless, the probability that the two virus groups meet *in vivo* is not so high, considering an AAV frequency of 1 ssDNA genome in 5×10^2 to 2×10^4 T lymphocytes of healthy individuals. Immunosuppression, encountered as transient episodes during diverse disease states, increases the probability of AAV activation, possibly by an altered T-cell response (45) or by herpesvirus coinfection, as hypothesized here.

Apart from AAV2, AAV5 was detected once in $CD3^+$ cells, though not exclusively. The presence of AAV5 in the $CD3^-$ fraction from the same donor may be taken as an indication of AAV5's variant cell tropism (51–53). The very rare previous detection of AAV5 DNA in human tissues further underlines its unique *in vivo* phenotype. Follow-up studies with larger cohorts of AAV-positive subjects will help to corroborate the data. The high prevalence of a spectrum of wild-type AAVs in PBMCs, though at low copy numbers, warrants attention during clinical gene therapy. Since for many applications enormous vector doses are injected into the bloodstream, coinfection of cells with

preexisting wild-type AAVs may lead to unwanted recombination between the genomes of AAV vectors and endogenous AAVs. Careful surveillance will be needed to minimize the hazard of unforeseen adverse events.

MATERIALS AND METHODS

Study protocol. This cross-sectional study was carried out according to Charité regulations for good scientific research conduct and was approved by the Ethics Commission of Charité Medical School. When required, informed consent was signed by the study participants. Blood samples from blood donors ($n = 243$) or immunosuppressed patients ($n = 41$) were randomly chosen. Although anonymous, the blood donors were mostly Berlin/Brandenburg residents, and the same is assumed for the immunosuppressed patients.

Plasmids. AAV plasmids carrying the *cap* gene of AAV1 to -3 or -5 to -13 (54, 55) were linearized with XbaI or SwaI and column purified (QIAquick PCR Purification kit; Qiagen), and serial dilutions (from 2×10^8 to 2 copies/μl) were prepared in H_2O or on the background of genomic DNA to determine PCR sensitivity. Plasmids for the AAV2 or AAV5 *cap* gene served as standards for quantitative PCR. For second-round amplification of infectious AAV in 293 cells, the plasmid pHelper (Stratagene) carrying helper functions from Ad5 was used. The yellow fluorescent protein (YFP)-expressing plasmid pYFP-C2 (Clontech) was used to monitor transfection efficiency.

Cells and viruses. HEK-293 cells and HeLa cells were grown as described previously (56). The HeLa cell clone HA16 carrying stably integrated, rescuable AAV2 genomes (57) served to evaluate the sensitivity of the secondary-infection assay. Adenovirus type 2 was propagated as described previously (56).

DNA isolation from human blood samples. EDTA blood samples (5 to 10 ml) collected for routine diagnostics, were centrifuged at 4,000 $\times$ *g* at room temperature for 5 min. DNA was extracted from 200 to 400 μl of buffy coat, using a QIAamp DNA Blood minikit (Qiagen). DNA from 10^7 HeLa cells was PCR tested in parallel to ensure purity of all the reagents used for DNA extraction.

AAV first-round PCR primer generation. AAVsero for (AAV2 nt 2350 to 2373) (5′-TAC AAG TAC CTC GGA CCC TTC AAC-3′) and AAVsero rev (AAV2 nt 2899 to 2878) (5′-TGG AAT CGC AAT GCC AAT TTC C-3′) span a conserved region of the AAV2 *cap* gene (Fig. 1A). For divergent AAV5, primer AAV5 for (AAV5 nt 2341 to 2364) (5′-GCT GCC TGG TTA TAA CTA TCT CGG-3′) was designed to amplify AAV5 combined with AAVsero rev. These primers produce PCR products of 532 to 553 bp, depending on the serotype of the AAV *cap* gene. AAV1 to -3 and -5 to -13 were sufficiently conserved to be amplified (Fig. 1).

Up to 1 μg of genomic DNA was amplified by PCR with primers AAVsero for, AAVsero rev, and AAV5 for (0.6 μM each); 0.22 mM deoxynucleoside triphosphates (dNTPs); and 5 U proofreading hot-start polymerase (Herculase; Stratagene) in reaction buffer to a final volume of 50 μl. The thermocycling profiles for PCR consisted of 1 cycle of 94°C for 2 min and 45 cycles of 94°C for 20 s, 61°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 7 min. Nontemplate (nuclease-free water) and total genomic DNAs from HeLa cells were used as negative controls.

Nested PCR. Nested primers that matched AAV1 to -3, AAV6 to -10, and AAV13 were generated (AAVsero nest for, 5′-AGA TAC GTC TTT TGG GGG CAA C-3′, and AAVsero nest rev, 5′-CGT TAT TGT CTG CCA TTG GTG C-3′). The expected PCR products ranged from 329 to 332 bp for different AAV serotypes. For AAV5, a separate nested primer pair was generated (AAV5 nest for, 5′-TCA AGT ACA ACC ACG CGG AC-3′, and AAV5 nest rev, 5′-ACT CCA TCG GCA CCT TGG TTA-3′), leading to an amplicon of 364 bp. A dynamic touchdown PCR was performed as follows: initial denaturation at 94°C for 2 min and 8 cycles of 94°C for 20 s, 63.5°C for 30 s (decreasing half a degree each cycle), and 72°C for 40 s, and then 10 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 40 s, followed by 10 cycles of 94°C for 20 s, 60°C for 30 s (decreasing half a degree each cycle), and 72°C for 50 s, and then 7 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 50 s, followed by a final extension step at 72°C for 7 min on a Bio-Rad MyCycler thermal cycler. For AAV5 nested PCR, the annealing temperature was 64°C for the first 8 cycles, and the steady annealing temperature in the last 7 cycles was 57°C.

Determination of AAV serotypes by DNA sequence analysis. PCR products were purified (GeneMatrix 3-in-1 basic DNA purification kit; EURx) and subjected to DNA sequencing (Eurofins Genomics, Ebersberg, Germany). Samples with mixed serotypes often resulted in superimposed DNA sequences. In these cases, the PCR products were cloned into pJET1.2 using the CloneJet PCR cloning kit (Thermo Scientific) prior to sequence analysis. The DNA sequences were aligned using NCBI BLAST (http://blast.ncbi.nlm.nih.gov).

Separation of leukocyte subpopulations by MACS. PBMCs were isolated from 50 ml freshly collected EDTA-blood samples using Ficoll-Paque Plus (GE Healthcare). After red blood cell lysis with ACK lysing buffer (Life Technologies), the PBMCs were washed with phosphate-buffered saline (PBS) and pelleted. The cells were counted, centrifuged, and resuspended in 80 μl wash buffer (PBS with 2 mM EDTA, pH 7.2) per 10^7 cells. $CD3^+$ and $CD3^-$ cells or $CD14^+$, $CD3^+$, and $CD14^-/CD3^-$ cells were isolated by two successive rounds of immunomagnetic bead selection, using CD3 microbeads or CD14 and CD3 microbeads, respectively, and LS columns (Miltenyi Biotec, Germany). The extracted DNA was analyzed by AAV PCR. AAV copy numbers were determined by real-time PCR. Samples and standards were preamplified by 20 cycles of AAV 1st-round PCR, as described above. A real-time LightCycler PCR was followed by 2.5 μl of 1st-round PCR product using 500 nM AAVsero nested primers in 20 μl reaction buffer (GoTaq qPCR master mix; Promega). The amplification conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s.

AAV infection assay. HEK293 cells were split 1:6 the day before transfection into 6-well or 12-well plates. The cells were cotransfected with 1.5 or 0.75 μg total genomic DNA from PBMCs and 0.5 or 0.25 μg pHelper (Ad helper functions) by the $CaPO_4$ precipitate technique as described previously (56). To facilitate transfection of high-molecular-weight genomic DNA, all samples were digested with EcoRV before transfection. The transfected cells were maintained for 9 days. To detect newly replicated AAV, HeLa cells seeded to 50% confluence on 6-cm dishes were coinfected with 500 μl of supernatants from transfected 293 cells and Ad2 (multiplicity of infection [MOI] = 20) and harvested 4 days postinfection (p.i.).

Western blot analysis. Cells from a 6-cm dish were harvested and divided in half. Western blots for the analysis of AAV Rep and Cap expression were prepared as described previously, using monoclonal antibody (MAb) 303.9 (anti-Rep) or MAb B1 (anti-VP) at a 1:10 dilution (58).

Quantification of newly replicated AAV particles. Viral DNA was isolated as described previously. Briefly, one-half of the cells from a 6-cm dish were lysed by freeze-thaw cycles, and crude extracts diluted in 500 μl supernatant were treated with benzonase to degrade nonpackaged DNA (58). After phenol-chloroform purification and ethanol precipitation, the DNA was resuspended in 20 μl 10 mM Tris. AAV genomes were quantified on a LightCycler in a 1:10 or 1:1,000 dilution using AAVsero for, AAV5 for, and AAVsero rev and GoTaq qPCR master mix (Promega). AAV5 rescue was determined by a AAV5-specific nested PCR. Samples and standards (2 μl) were preamplified on a block cycler for 20 cycles with primers AAV5 for and AAVsero rev using herculase, as described above. Quantification was performed on a LightCycler using 1st-round PCR samples as templates (1/50 volume) with primers AAV5 nest for and AAV5 nest rev; 10^6 copies of pTAV2.0 (AAV2) served as a negative control.

Detection of AAV helper viruses. DNA samples were PCR analyzed by IKDT (Berlin, Germany) to detect HCMV and HHV-6 genomes as described previously (40).

Statistics. Statistical analysis was performed using a χ^2 test or Fisher's exact test.

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